Exploring the Role in Translation Quality Control of Cytoplasmic Phenylalanyl-tRNA Synthetase

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Abstract

A critical step in gene expression is the faithful translation of protein from messenger RNA. This process relies on the attachment of specifically paired amino acids to their respective tRNAs by aminoacyl-tRNA synthetases (aaRSs). To ensure high accuracy of tRNA aminoacylation, aaRSs rely on maintaining high substrate specificity and editing activity. While editing significantly decreases the frequency of errors during aa-tRNA synthesis *in vitro*, many details of the reaction remain unknown. Eukaryotic organisms contain two distinct phenylalanyl-tRNA synthetases (PheRS), a cytoplasmic (cytoPheRS) and a mitochondrial form (mitotPheRS). *Saccharomyces cerevisiae* cytoPheRS has lost some amino acid specificity but maintains an editing acitivity, while the mitoPheRS maintains amino acid specificity but has lost editing activity. To investigate the impact of editing activity on the cell, *S. cerevisiae* cytoPheRS was engineered to have a high level of amino acid specifity with and without editing activity. To do this the wild-type *FRS2* gene, which encodes the cytoPheRS α-subunit and contains the active site, was cloned onto a centromeric plasmid and used to complement a heterozygous *FRS2* deletion (*FRS2*/*frs2Δ*). The *FRS2* wild-type gene product was found to be functional *in vivo*. The *FRS2* specificity mutant (*frs2-1*) was also cloned and determined to be functional *in vivo* as well. The functional *frs2-1* strain will next be used to complement an editing defective strain (*frs1-1*) which shows an amino acid dependent growth defect. This work will help clarify the impact of amino acid specificity and post-transfer editing on cellular physiology.

Introduction

The process by which information from a gene is used to synthesize a functional gene product and subsequently the macromolecular machinery necessary for life is termed gene expression. One of the critical steps in gene expression is the faithful translation of protein from messenger RNA. The fidelity of translation is determined by two events: the synthesis of cognate amino acid:tRNA pairs by aminoacyl-tRNA synthetases (aaRSs) and accurate selection of aminoacyl-tRNAs (aa-tRNAs) by the ribosome. The amino acid first binds to the active site of the aaRS and is activated by ATP, leading to the formation of an aminoacyl-adenylate and the release of an inorganic pyrophosphate (Figure 1). The amino acid is then transferred to the terminal adenosine of the tRNA, leading to the formation of the aa-tRNA and the release of AMP. These aa-tRNAs, once formed by aaRSs, are delivered to the ribosome by EF-Tu for protein synthesis. EF-Tu, or elongation factor thermo unstable, delivers the aminoacly-tRNA (aa-tRNA) to the A site of the ribosome and, if the pairing between the tRNA and the mRNA is correct, the amino acid is attached to the growing polypeptide (protein) chain.

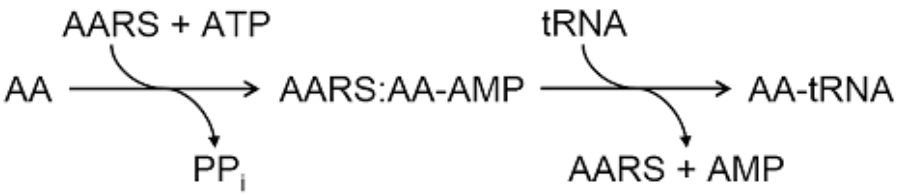

Figure 1. Synthesis of aminoacyl-tRNA by aminoacyl-tRNA synthetases. Two-step reaction of aminoacyl-tRNA synthesis by aminoacyl-tRNA synthetases. An aminoacyl-adenylate is first formed by the activation of the amino acid with ATP. The aminoacyl group is then transferred to the 3' end of the tRNA and the aminoacyl-tRNA is released.

Errors in aminoacylation may result in the incorporation of the incorrect amino acid during protein synthesis. To ensure a high accuracy of aminoacylation, aaRSs rely on maintaining high substrate specificity. Amino acid specificity is achieved by aaRSs through the preferential binding of cognate amino acids over non-cognate amino acids as well as the editing

of non-cognate amino acids (Ibba and Söll, 2000). Most eukaryotic organisms contain two distinct aaRS genes, one that encodes for an aaRS in the cytoplasm and the other in the mitochondria (Figure 2). *Saccharomyces cerevisiae* cytoplasmic phenylalanyl-tRNA synthetase (cytoPheRS) has lost some amino acid specificity but maintains an editing acitivity, while the mitochondrial phenylalanyl-tRNA synthetase (mitoPheRS) maintains amino acid specificity but has lost editing activity (Roy *et al*., 2005).

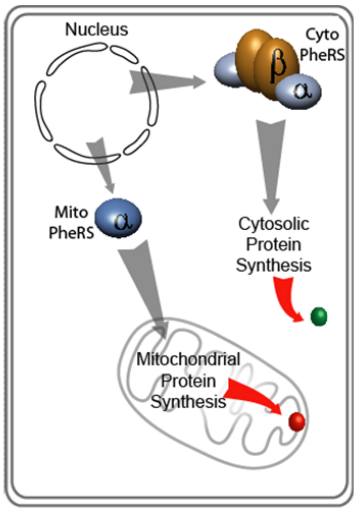

Figure 2. Sub-cellular targeting of PheRS. Mitochondrial and cytosolic forms of PheRS are both encoded in the nucleus by separate genes. After exiting the nucleus mtPheRS is imported into the mitochondria.

CytoPheRS is a multidomain $(\alpha\beta)_2$ heterotetrameric enzyme made of two αβ dimers. The α-subunit contains the active site of the enzyme where the incoming amino acid is activated by ATP to form an aminoacyl-adenylate followed by attachment to the tRNA 3'-end, while the β-subunit contains an editing domain. Both of these functions, the amino acid specificity and the editing activity, function to maintain quality control of cytoplasmic translation.

Mutations in editing have been shown to adversely affect cell growth and viability *in vivo*, particularly under cell stress (Nangle *et al*., 2002). Slight perturbations in editing have been shown to result in severe phenotypic effects and in the absence of editing, misactivation by

aaRSs can produce levels of misincorporation that eventually increase the DNA mutation rate (Bacher and Schimmel, 2007). However, editing is not universally conserved in aaRSs. For instance, cytoplasmic proline-tRNA synthetase (ProRSs) from archaea and higher eukaryotes lack a posttransfer editing domain, while some other eukaryotic species have obsolete editing domains (Stern *et al*., 2007). Eukaryotic mitochondrial phenylalanyl-tRNA synthetase (PheRS) and human mitochondrial leucine-tRNA synthetase (LeuRS) have completely lost their editing activity and in yeast mitoLeuRS editing is unnecessary; instead, these aaRSs rely solely on a high level of amino acid specificity (Roy *et al*., 2005; Karkhanis *et al*., 2006). This loss of editing activity is restricted to the organellar forms of these synthetases, and the continued need for editing by their cytosolic complements suggests that there are different controls placed on the fidelity of protein synthesis in various subcellular compartments (Yao *et al*., 2008). While editing significantly decreases the frequency of errors during aa-tRNA synthesis *in vitro*, many details of the reaction remain unknown.

To investigate the impact of editing activity on the cell, *Saccharomyces cerevisiae* cytoplasmic phenylalanyl-tRNA synthetase (cytoPheRS) was engineered to enhance substrate specificity as a means to make the active site more specific for Phe. To do this the wild-type FRS2 gene, which encodes the cytoPheRS α-subunit and is the active site (Figure 3), was cloned onto a centromeric plasmid and used to complement a heterozygous FRS2 deletion (*frs2Δ*). The FRS2 wild-type gene product was found to be functional *in vivo*. The FRS2 specificity mutant (*frs2-1*) was also cloned and the gene product was determined to be functional *in vivo* as well. The functional *frs2-1* strain will next be used to complement an editing defective strain which shows an amino acid dependent growth defect. This work will help clarify the impact of amino acid selectivity and post-transfer editing on cellular physiology.

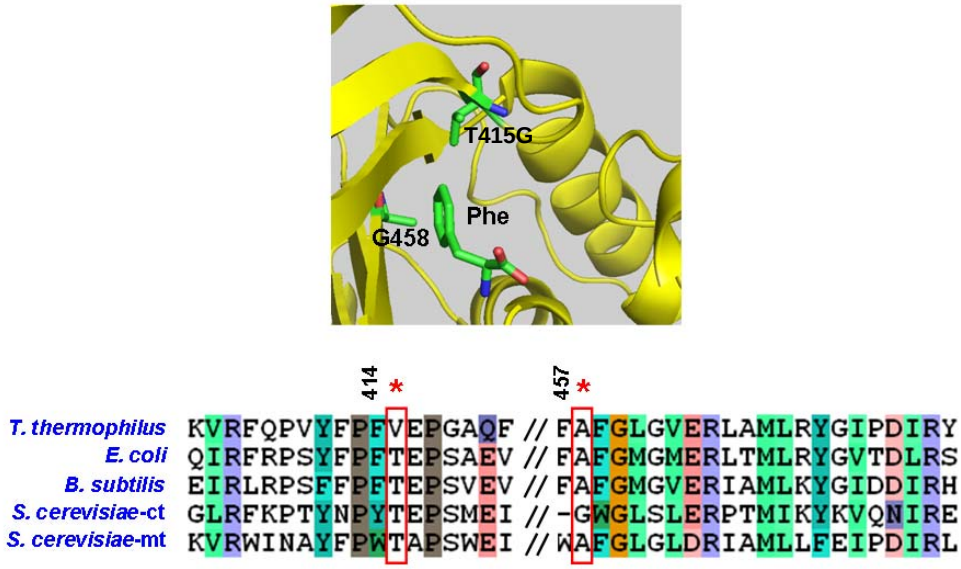

Figure 3. PheRS active site. (A) *T. thermophius* PheRS with Phe at the active site with cytoPheRS numbering. (B) Specificity determinants highlighted in a PheRS sequence alignment (cytoPheRS numbers) (adapted from Reynolds *et al*., 2010).

Materials and Methods

Polymerase chain reaction was used to amplify wild-type FRS2 (encoding the cytoPheRS α-subunit) gene sequence and regulatory regions from *S. cerevisiae* BY4743. The gel-purified 2700 bp PCR product was then cloned into the centromeric plasmid, pFL36, using In-Fusion cloning (Zhu *et al*., 2007). The resulting plasmid (pFL36-*FRS2*) was sequenced to confirm proper cloning and lack of mutations. The pFL36-*FRS2* was transformed into the heterozygous *FRS2* deletion (*frs2Δ*) *S. cerevisiae* strain BY4743 YFL022C (MATa/MATα, *his3Δ1*/*his3Δ1*, *leu2Δ0*/ *leu2Δ0*, *lys2Δ0*/*LYS2*, *MET15*/*met15Δ0*, *ura3Δ0*/*ura3Δ0*, *FRS2*/*frs2Δ*) (ATCC). The resulting strain was sporulated on sporulation medium (1 % CH_3COOK, 0.1 % yeast extract, 0.05 % glucose) and spores dissected onto yeast extract/peptone/dextrose (YPD). The dissected spores were grown at 37 °C and replicated onto YPD with 200 μg/mL Geneticin (G418), to select for spores containing the *frs2* knock-out, and complete supplement media minus leucine (CSM –Leu; Sunrise Science Products), to select for spores containing the pFL36-*FRS2* plasmid, and grown for 48 hours at 37 °C.

In vivo site directed mutagenesis was performed with pFL36-*FRS2* to introduce the G458A mutation (*frs2-1)* and the resulting plasmid (pFL36-*frs2-1*) was sequenced to confirm the G458A mutation. The purified pFL36-*frs2-1* plasmid was then transformed into the heterozygous *FRS2* deletion (*frs2Δ*) *S. cerevisiae* strain BY4743 YFL022C and the resulting strain was sporulated and spores dissected onto YPD as above. The dissected spores were grown for 48 hours at 37 °C, replicated onto G418 to select for spores containing the *frs2*Δ and CSM minus leucine to select for spores containing the pFL36-*FRS2* plasmid. Replica plates were grown for 48 hours at 37 °C.

The cytoPheRS α and β subunits, encoded by the *FRS2* and *FRS1* genes, respectively, were expressed in tandem from pQE31-FRS-sc (producing His6-tagged WT cytoPheRS) in *E. coli* (Roy *et al*., 2005). The T415G and G458A mutations were introduced into the cytoPheRS α-subunit using Quikchange site-directed mutagenesis. His6-tagged proteins were purified on nickel-nitrilotriacetic acid-agarose by standard procedures.

Results

CytoPheRS G458A shows increased amino acid specificity and decreased catalytic efficiency. However, the double mutant T415G/G458A showed less than 5% activity.

	Phe			Tyr			Specificity
	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($s^{-1}μM^{-1}$)	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($s^{-1}μM^{-1}$)	
Cytosolic							
WT	3±0.1	603±41	194±14	637±308	184±36	0.4±0.2	480
G458A	233±27	464±37	0.2±0.3	3000±1200	0.6±0.25	0.0002±0.0001	10000

Table 1. CytoPheRS has low amino acid specificity. Steady state kinetic constants of cytosolic wild type and binding site mutant PheRSs for ATP-[^{32}P]PPi exchange (performed by NMR with the assistance of SR).

Wild-type cytoPheRS FRS2, pFL36-*FRS2*, shows growth on YPD and G418 demonstrating that pFL36-*FRS2* is able to complement the *frs2-1Δ* and support growth (Figure 4). *FRS2* is an essential gene necessary for cell growth and survival. Growth of all four of the wild-type cytoPheRS *FRS2*, pFL36-*FRS2*, spores on YPD indicates that each spore had a functional *FRS2* gene. In these spores, the functional *FRS2* gene could be present as solely wild-type *FRS2* gene on the chromosome or as wild-type *FRS2* on the chromosome in combination with the plasmid, pFL36-*FRS2*, or as solely on the plasmid, pFL36-*FRS2*, in combination with the knock-out *frs2Δ* that contains a *kanMX* cassette.

To select for spores that contained the knock-out *frs2Δ* and the plasmid, pFL36-*FRS2*, the tetrads were replica-plated onto G418 plates. G418 is an antibiotic whose resistance is conferred by *kanMX*, which was put in place of the wild-type *FRS2* gene in the knock-out. As a centromeric plasmid pFL36 replicates similar to natural chromosomes, meaning that each tetrad should have received a copy of the pFL36-*FRS2* plasmid. However, as the *S. cerevisiae* BY4743 YFL022C strain was heterozygous for the *FRS2* gene, only those tetrads that received the plasmid and had the knock-out with the *kanMX* cassette should have been able to grow on G418.

Growth of mutant G458A cytoPheRS *frs2-1*, pFL36-*frs2-1*, on YPD and G418 shows that pFL36-*frs2-1* is also able to complement *frs2-1Δ* and support growth.

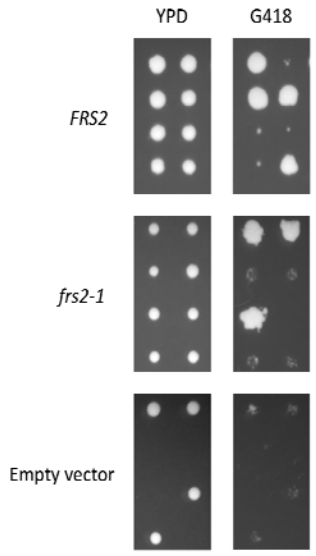

Figure 4. CytoPheRS complementation. Dissection of tetrads from *S. cerevisiae FRS2*/*frs2*Δ complemented with (A) pFL36-*FRS2*, (B) pFL36-*frs2-1* and (C) pFL36 onto YPD and replica plated onto G418.

Discussion

S. cerevisiae cytoplasmic phenylalanyl-tRNA synthetase (cytoPheRS) has been shown to have low amino acid specificity, as approximately one Tyr is activated for every ~480 Phe activated (Table 1). However, cytoPheRS does have an editing activity which compensates for this lack of selectivity. Changing the selectivity of the active site by changing the Glycine residue at position 458 into an Alanine residue nevertheless enhances the selectivity of the α-subunit of cytoPheRS so that only one Tyr is activated for every ~10000 Phe activated (Table 1). Based on the steady-state kinetic constants of cytosolic PheRSs for ATP-[^{32}P]PPi exchange, the catalytic efficiency of the G458A cytoPheRS mutant was so low that *in vivo* functionality was uncertain. However, growth of both the wild-type pFL36-*FRS2* and the mutant pFL36-*frs2-1* tetrads show that specificity of the active site can be enhanced and still functional *in vivo*. Other aaRS mutants (T415G/G458A) showed less than 5% activity, making the cytoPheRS G458A mutant the best system to study the need for specificity versus editing activity.

The need for specificity versus editing activity will be determined by complementing an editing deficient strain of the cytoPheRS with pFL36-*frs2-1*. The editing deficient strain shows no growth phenotype on rich media where the ratio between Phe and Tyr is 4:1 (Reynolds *et al*., 2010). However, the editing deficient strain shows a growth phenotype on minimal media where the Phe to Tyr ratio is only 1:1.1 (Reynolds & M Ibba, unpublished data). Using the pFL36-*frs2-1* plasmid, the editing deficient strain will be complemented and grown on minimal media to see if the wild-type growth phenotype can be recovered. However, as the G458A mutation result in a K_M defect for Phe and thus is sensitive to amino acid concentrations, growth of the strain carrying the pFL36-*frs2-1* in minimal media, where the free phenylalanine concentration is reduced is an important test of function. Preliminary results suggest that G458A has a slow growth phenotype on minimal media (data not shown). These experiments provide the groundwork for the further clarification of the impact and importance of amino acid specificity and post-transfer editing by aaRSs on cellular physiology.

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Cell-specific differences in the requirements for translation quality control

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Protein synthesis has an overall error rate of approximately 10^{-4} for each mRNA codon translated. The fidelity of translation is mainly determined by two events: synthesis of cognate amino acid:tRNA pairs by aminoacyl-tRNA synthetases (aaRSs) and accurate selection of aminoacyl-tRNAs (aa-tRNAs) by the ribosome. To ensure faithful aa-tRNA synthesis, many aaRSs employ a proofreading ("editing") activity, such as phenylalanyl-tRNA synthetases (PheRS) that hydrolyze mischarged Tyr-$tRNA^{Phe}$. Eukaryotes maintain two distinct PheRS enzymes, a cytoplasmic (ctPheRS) and an organellar form. CtPheRS is similar to bacterial enzymes in that it consists of a heterotetramer in which the α-subunits contain the active site and the β-subunits harbor the editing site. In contrast, mitochondrial PheRS (mtPheRS) is an α-subunit monomer that does not edit Tyr- $tRNA^{Phe}$, and a comparable transacting activity does not exist in organelles. Although mtPheRS does not edit, it is extremely specific as only one Tyr-$tRNA^{Phe}$ is synthesized for every ~7; 300 Phe- $tRNA^{Phe}$, compatible with an error rate in translation of ~10^{-4}. When the error rate of mtPheRS was increased 17-fold, the corresponding strain could not grow on respiratory media and the mitochondrial genome was rapidly lost. In contrast, error-prone mtPheRS, editing-deficient ctPheRS, and their wildtype counterparts all supported cytoplasmic protein synthesis and cell growth. These striking differences reveal unexpectedly divergent requirements for quality control in different cell compartments and suggest that the limits of translational accuracy may be largely determined by cellular physiology.

aminoacyl-tRNA synthetase - protein synthesis - tRNA

Introduction

Typical error rates for individual steps in gene maintenance and expression range from 10^{-8} for DNA replication (1) to 10^{-5} for mRNA transcription (2) and 10^{-4} for mRNA translation (3). These low error rates are achieved through high substrate specificity augmented by monitoring and proofreading of erroneous product synthesis, ensuring a high level of quality control. Whereas each cellular quality control mechanism has optimized its own level of specificity, translation as a whole limits misincorporation of the incorrect amino acid to one per 10,000 mRNA codons (3, 4). The fidelity of translation is determined by multiple events including synthesis of cognate amino acid:tRNA pairs by aminoacyl-tRNA synthetases (aaRSs), binding and delivery of aminoacyl-tRNAs (aa-tRNAs) to the ribosome by elongation factors, and accurate selection of aa-tRNAs by the ribosome (5, 6). Despite the widely held notion that limits on quality control, and tolerable error rates, are a fundamental aspect of all cells, recent studies suggest that wide disparities exist between different cell types particularly during translation. For example, partial ablation of aaRS proofreading in mice had no discernible effect on early growth and development but specifically impacted neuronal cells leading to ataxia and neurodegeneration in older animals (7).

During translation of the genetic code, aaRSs provide a critical step in quality control by preferentially selecting cognate pairs of tRNAs and amino acids while discriminating against

near- and noncognate molecules. The unique combinations of sequences and structures found in particular tRNAs allow cognate molecules to be specifically selected out of the large cellular pool of similar molecules without recourse to proofreading (5, 8). Amino acids, by contrast, present a much more challenging problem for their cognate aaRS. The 20 naturally occurring amino acids do not display a sufficiently diverse range of functional groups that would allow aaRSs to discriminate between them with a level of accuracy consistent with the error rate assigned to translation (9). To prevent degeneracy of the genetic code by the infiltration of near-cognate amino acids, a number of proofreading activities are employed by aaRSs. These editing activities can occur through the hydrolysis of misactivated aminoacyladenylates (pretransfer editing) and/or through the hydrolysis of mischarged aa-tRNAs (posttransfer editing) (10). For example, the editing activities of phenylalanyl-tRNA synthetase (PheRS) prevent the delivery of Tyr- $tRNA^{Phe}$ to the ribosome and protect against the mistranslation of Phe codons as Tyr (11, 12).

The eubacterial, archaeal, and eukaryotic cytoplasmic PheRSs are heterotetrameric proteins composed of two α/β-heterodimers in which the α-subunits contain the active site and the β-subunits contain the editing site (13, 14). In eukaryotic cells separate translational systems are maintained in the cytoplasm and organelles, and aaRSs from both are encoded in the nucleus, with organelle forms synthesized as preproteins, which are then imported and processed (15). The mitochondrial form of PheRS (mtPheRS), as well as the chloroplast form, is an α-subunit monomer that lacks a recognizable editing domain, consistent with the absence of editing in some other mitochondrial aaRSs (16–18). Whereas ctPheRS and eubacterial PheRS possess a posttransfer editing activity against misacylated species, mtPheRS does not have the ability to edit mischarged Tyr- $tRNA^{Phe}$, nor do mitochondria contain any trans-editing activity able to compensate for this deficiency (19). We now show that in *Saccharomyces cerevisiae* mtPheRS relies solely on a high level of specificity for Phe over Tyr for quality control of aminoacylation. A decrease in the amino acid specificity of PheRS blocked mitochondrial biogenesis but did not affect normal growth when tested in either the yeast cytoplasm or a bacterial model system. These data reveal strikingly different requirements for aaRS-mediated translation quality control in various cellular environments.

Results

Amino Acid Specificity of PheRS. Previous studies of *Escherichia coli* PheRS showed that the replacement αA294G enlarged the amino acid binding pocket of the active site of the enzyme, allowing tRNA aminoacylation with *para*-halogenated Phe analogs (20). Steady-state kinetic analyses confirmed the role of this residue in *E. coli* PheRS quality control, the αA294G variant showing almost a 100-fold loss in specificity for Phe over Tyr (Table 1). To investigate the conservation of this specificity determinant, 1,179 PheRS α-subunit sequences (877 from eubacteria, 23 from archaea, and 160 and 119 cytosolic and mitochondrial eukaryotic, respectively) were aligned with ClustalX (21), guided by the three-dimensional structure of *Thermus thermophilus* PheRS [Fig. 1A (22)], and refined manually. αAla294 was found conserved at the equivalent position in all sequences except for 48 cytoplasmic PheRSs from 33 genera of eukaryotes that contained a Gly residue (Fig. 1B). This natural substitution of Ala by Gly showed no obvious phylogenetic distribution and was observed in all fungi (not in microsporidia), in the primitive eukaryote *Trichoplax adhaerens*, and also in some higher eukaryotes. The lack of conservation of the αAla294 specificity determinant among several PheRSs prompted us to compare the substrate specificity of the S. cerevisiae mitochondrial (αAla333) and Cytoplasmic (αGly458) PheRSs, the latter of which contain a natural Ala to Gly substitution. The ctPheRS was 5 times more efficient (as reflected in kcat/KM) than the mtPheRS

with respect to Phe activation and 80 times more efficient with respect to Tyractivation (Table 1). These differences in amino acid activation kinetics revealed that the specificity for Phe over Tyr is 15-fold higher for mtPheRS than it is for ctPheRS. To further investigate the ability of the αAla294 equivalent residues to confer specificity during Phe/Tyr discrimination in *S. cerevisiae,* mtPheRS was engineered through an A333G replacement whereas the converse change, G458A, was made in ctPheRS. MtPheRS A333G showed a 17-fold reduced specificity for Tyr in vitro compared to wildtype mtPheRS, whereas ctPheRS αG458A displayed a 20-fold increase compared to wild-type, confirming the critical role of this residue in quality control during amino acid activation (Table 1).

Table 1. Steady-state kinetic constants for ATP-[^{32}P]PP$_i$ exchange for cytosolic and mitochondrial wild-type and variant PheRS from *S. cerevisiae* and *E. coli*.

	Phe			Tyr			Specificity
	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M(s^{-1}/μM^{-1})	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M(s^{-1}/μM^{-1})	
E. coli							
WT	2±0.	199±2	110±4	2200±70	35±	0.016±0.00	6800
A294G	4.5±1.	185±1	45±1	320±8	185±2	0.6±0.0	75
Mitochondrial							
WT	5±0.	180±7.	35±0.	1155±16	5±0.	0.005±0.000	7300
A333G	17±	140±1	8±0.	660±5	12.5±0.	0.02±0.00	426
Cytosolic							
WT	3±0.	603±4	194±1	637±30	184±3	0.4±0.	485
G458A	233±2	464±3	0.2±0.	3000±120	0.6±0.2	0.0002±0.000	10000

Mitochondrial PheRS Specificity is Essential for Respiratory Function. MtPheRS A333G displayed comparable substrate specificity to wild-type ctPheRS but lacked the latter protein's ability to edit Tyr- tRNA^{Phe}, which provided a means to investigate the importance of specificity for translational quality control in vivo by replacing wild-type mtPheRS with the A333G variant. To exclude possible indirect effects of the A333G replacement resulting from changes in secondary structure, CD spectral analysis was performed. Wild-type and mutant mtPheRS proteins showed nearly identical far-UV CD spectra, suggesting A333G does not induce major changes in global secondary structure (Fig 1C).

To test the effect of changing amino acid specificity in vivo, a chromosomal A333G replacement in *MSF1* (encoding mtPheRS) was constructed in a haploid yeast strain resulting in the *msf1-1* allele, and this strain was then crossed to a haploid wild-type to ensure the presence of fully functional mitochondria with an intact genome. Dissection of the heterozygous diploid *MSF1/msf1-1* strain on fermentative medium (glucose) resulted in growth of all spores in each tetrad and replica plating onto media requiring respiratory function (ethanol plus glycerol) resulted in a nogrowth phenotype that segregated 2:2. On fermentative media both the msf1-1 and *msf1Δ* strains showed a reduced level of growth compared to *MSF1* (Fig. 2A and B). However, on respiratory media neither the *msf1-1* nor *msf1Δ* strain showed any growth (Fig 2B). To determine if the mitochondria are unable to respire from the onset or the ability to respire is lost with time, *MSF1/msf1-1* was sporulated and dissected directly onto respiratory medium (Fig.

2C). While *msf1-1* spores were able to germinate, growth was quickly arrested. It is possible that the limited growth seen is a result of the presence of both wild-type and mutant mtPheRS in the mitochondria at the onset of germination. Thus, the mitochondria may be able to respire for a very short time with the mitochondrial genome becoming unstable as a result of protein turnover and the increase in population of mutant mtPheRS present.

To investigate the presence or absence of the mitochondrial genome, *msf1-1* was crossed with a *MSF1 rho*^{0} strain that encodes a wild-type mtPheRS but is devoid of the mitochondrial genome. If the *msf1-1* strain is limited in respiratory activity because of the mutant mtPheRS but retains an intact mitochondrial genome, crossing with a *MSF1 rho*^{0} strain would result in a diploid with fully functional mitochondria, imparting the ability to grow on media requiring respiration. When tetrads from *MSF1/msf1-1* were dissected, the spores allowed to germinate for 36 h, and then crossed with the *MSF1 rho*^{0} strain to monitor the loss of the mitochondrial genome, 84 out of 100 *msf1-1* spores maintained their mitochondrial genome, whereas 16 of 100 spores had lost their mitochondrial genome at the time of crossing. However, after germination, if *msf1-1* was grown in an overnight liquid culture before crossing, the *MSF1 rho*^{0} strain was unable to complement *msf1-1*, indicating a complete loss of the mitochondrial genome from *msf1-1*. These results demonstrate that reducing the specificity of mtPheRS is sufficient to destabilize the mitochondrial genome, which is then lost over time. This result is in agreement with previous findings in yeast where mutations inactivating mitochondrial translation result in mitochondrial genome instability (23).

PheRS Quality Control Mechanisms are not Essential in E. coli or S. cerevisiae Cytoplasm. Loss of amino acid specificity encoded by the *MSF1* gene resulted in ablation of mitochondrial biogenesis in *S. cerevisiae*, prompting us to investigate the requirements for this quality control mechanism in other cell types. In order to provide a direct comparison to mitochondria, *E. coli* was chosen as a model system because it has previously been demonstrated that mtPheRS can efficiently aminoacylate *E. coli* tRNA^{Phe} (24, 25). To determine if loss of specificity in PheRS has a similar impact on cellular physiology in mitochondria and bacteria, we attempted to complement the *E. coli* strain NP37, which encodes a temperature-sensitive PheRS variant (26). NP37 was transformed with plasmids expressing *MSF1*, *msf1-1*, *FARS2* (encoding human mtPheRS), or *fars2-1* (A308G) and the resulting transformants grown at permissive or restrictive temperature (Fig. 3A and B). Both yeast and human wild-type mtPheRS, and the corresponding compromised specificity mutants, rescued the growth phenotype at a restrictive temperature. To determine if growth at the restrictive temperature resulted from retention of *E. coli* PheRS editing activity at 42 °C, Tyr- tRNA^{Phe} hydrolysis activity was measured in cell-free extracts (Fig. 3C). *NP37/fars2-1* extracts displayed editing activity when prepared from cells grown at 30 °C but not at 42 °C, confirming that *E. coli* NP37 lacks both PheRS editing and aminoacylation activities at the restrictive temperature.

To further compare requirements for quality control in different cellular compartments, the effect of ablating the Tyr- tRNA^{Phe} editing activity of ctPheRS was investigated in vivo. The yeast ctPheRS variant βD243A, which lacks editing activity (19), was inserted into the low copy-number centromeric plasmid pFL36, resulting in the plasmid pFL36-frs1-1, which was then used to transform the diploid strain YLR060W BY4743 (FRS1/frs1Δ). The resulting strains were sporulated, dissected onto yeast extract/peptone/dextrose (YPD), and replica plated onto G418 and complete supplement media minus leucine (CSM-Leu). The 36 resulting complete tetrads all showed 2:2 segregation on G418 and showed growth on CSM-Leu, confirming the viability of the *frs1Δ* strain complemented with *frs1-1* (Fig. 4A). Enzymatic Tyr- tRNA^{Phe} hydrolysis activity was not observed in extracts prepared from *frs2Δ* pFL36-*frs1-1* cells, indicating that ctPheRS

editing activity is absent in this strain (Fig. 4B). Taken together, these findings suggest that neither high amino acid specificity nor nearcognate amino acid editing by PheRS are required for *E. coli* or *S. cerevisiae* cytoplasmic growth under normal conditions.

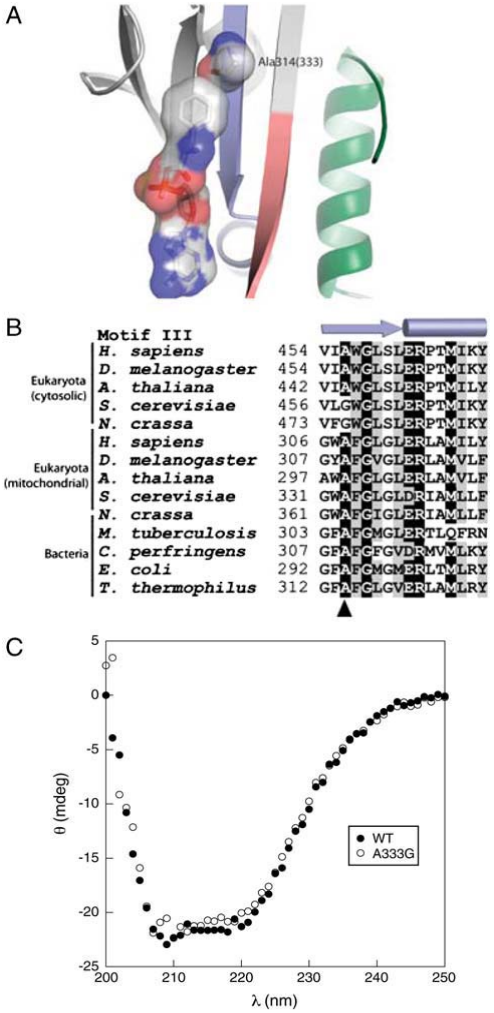

Fig. 1. Active site specificity of mitochondrial and cytosolic PheRS. (A) Structure of active site of the α-subunit of *T. thermophilus* PheRS in complex with phenylalanyl-adenylate (22). The optimal size of the Phe binding pocket is provided by residue Ala314 (residue A333 of *S. cerevisiae* mitochondrial PheRS). Motifs 1, 2, and 3 forming the active site of PheRS are colored in green, red, and blue, respectively. (B) Alignment summary of PheRS motif 3. The secondary structure elements of motif 3 are indicated on the top of the alignment, and position Ala314 is indicated by the arrowhead. Residues displaying more than 85% identity or similarity are depicted on black or gray background, respectively. (C) Secondary structural analysis of mtPheRS. Circular dichroism was measured at 25 °C with 5 μM samples of S. cerevisiae wild-type mtPheRS or the A333G variant.

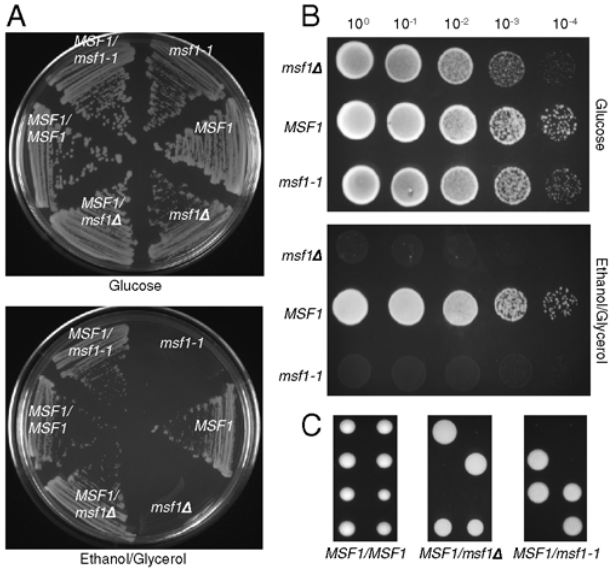

Fig. 2. Growth of *S. cerevisiae msf1*-1. (A) Streak plates showing growth of diploid and haploid strains on YPDA and ethanol plus glycerol medium. (B) Serial dilutions of strains obtained from dissection of *MSF1/msf1-1* on YPDA; *MSF1 msf1-1*, and *msf1Δ* on YPDA and ethanol plus glycerol medium. Plates were incubated at 30 °C for 3 days. (C) Dissection of tetrads from *MSF1/ MSF1*, *MSF1/msf1Δ*, and *MSF1/msf1-1* strains on ethanol plus glycerol medium. Plates were incubated at 30 °C for 3 (*MSF1/MSF1*) or 4 days (*MSF1/msf1Δ* and *MSF1/msf1-1*).

Discussion

Amino Acid Selectivity of aaRSs. Early studies on protein synthesis suggested overall error rates of around one misincorporated valine per 3,000 isoleucine codons translated (4). Upon the basis of this and other studies, it was proposed that aaRSs would generally need to achieve at least 3,000-fold selectivity for cognate over near-cognate amino acids to avoid elevating the error rate of protein synthesis (27). Amino acid selectivity takes into account both aaRS specificity and the relative cellular concentrations of the corresponding cognate and near-cognate substrates. aaRSs which did not show an overall selectivity above 1 in 3,000 were predicted to require some form of proofreading to maintain sufficient accuracy during aa-tRNA synthesis, as borne out by the

discovery of numerous editing mechanisms (10, 28). Biochemical characterization of the yeast PheRSs is consistent with this model; ctPheRS, an editing enzyme, displays 480-fold specificity for Phe over Tyr, which corresponds to a selectivity ranging from 1 in 400–2,000 depending on growth conditions (29). MtPheRS, which does not edit, displays 7,300-fold specificity for Phe over Tyr, which corresponds to a selectivity of 11,700 upon the basis of mammalian mitochondrial amino acid pools (30). *E. coli* PheRS displays a comparable predicted selectivity of 1 in 14,400 for Phe under standard conditions (31) while also retaining a robust editing activity (Table 2). The three PheRSs characterized here are all predicted to be equally effective in providing a level of cognate amino acid selectivity consistent with protein synthesis error rates lower than 1 in 3,000; the significant divergence in exactly how each enzyme prevents Tyr-tRNA^{Phe} synthesis may reflect adaptations to specific cellular conditions, which could place greatly different demands on the accuracy of translation. How amino acid pools vary under different conditions in diverse cellular environments, and how this changes the demands on aaRS quality control, remains to be determined.

Table 2. Predicted selectivity of PheRS in different cells and compartments.

Cell type	PheRS	Cellular Phe:Tyr*	Specificity Phe/Tyr†	Selectivity Phe/Tyr‡	Tyr-tRNA^{Phe} editing	Viable in vivo
Yeast mitochondria						
	mtPheRS	1.6:1§	7,300	11,700	No	Yes
	A333G mtPheRS	1.6:1	420	690	No	No
Yeast cytoplasm						
	ctPheRS	4.6:1¶	485	2,200	Yes	Yes
	D243A ctPheRS	4.6:1	485	2,200	No	Yes
E. coli						
	E. coli PheRS	1.9:1‖	6,800	14,400	Yes	Yes
	A333G mtPheRS	1.9:1	420	890	No	Yes

*Ratio of concentrations of free Phe and Tyr.
†See Table 1.
‡Selectivity was defined as (specificity) × ([Phe]/[Tyr]), as applied to amino acid activation, but does not take into account possible posttransfer editing.
§Estimate based on rat mitochondria (30).
¶Total cellular amino acid pools (29).
‖(31).

Divergent Cellular Requirements for Quality Control During Translation. The mitochondrial form of PheRS does not contain the β domain responsible for posttransfer editing and relies instead on a high level of amino acid specificity to maintain the fidelity of aminoacylation. This reliance on specificity alone is similar to mtLeuRS and certain prolyl-tRNA synthetases, all of which are highly specific and do not require editing, in contrast to their respective counterparts in *E. coli*, which both display robust editing activities (17, 18, 32, 33). The detrimental effects on the cell of reducing specificity for Phe confirmed the reliance of mtPheRS function on accurate amino acid recognition. Introduction of the A333G mtPheRS variant reduced the selectivity for Phe over Tyr to ~700:1, below the quality control "threshold" of 3; 000:1, a level of accuracy that proved to be too low to sustain mitochondrial biogenesis. Several proteins of the respiratory chain complex are synthesized within mitochondria (34, 35), and posttranslational quality control is used extensively to prevent dysfunction of the organelle (36). Our data now indicate that aaRS specificity is also an indispensable component of the quality control machinery in mitochondria.

The need for a high level of substrate specificity in order for PheRS to function properly in translation was not observed in bacteria or the yeast cytoplasm. Despite a specificity for Phe over Tyr of only ~420:1, A333G mtPheRS was able to support growth of *E. coli* on complete media. βD243A ctPheRS has a similar specificity, cannot edit mischarged tRNAPhe, and also supports cytoplasmic protein synthesis and growth. This ability to tolerate low specificity is in sharp contrast to the requirement for high amino acid specificity in mitochondria and provides direct evidence that certain cell types differ with respect to their requirements for quality control

during translation. The notion that translation quality control requirements are cell-specific is supported by other recent in vivo studies using a mouse model. Lee *et al*. found that a missense mutation in the editing site of AlaRS resulted in the accumulation of misfolded proteins and cell death in terminally differentiated Purkinje neuronal cells. Only these nondividing cells, which contain an extremely high concentration of protein, show this phenotype while all other cells appear normal. When taken together with these findings, our data now clearly indicate that the requirements for quality control during translation vary greatly depending on cellular physiology. Given that both *E. coli* and the yeast cytoplasm can tolerate a low specificity for Phe over Tyr yet contain proofreading PheRSs, it is also apparent that the true role of aaRS editing in the cell still remains to be fully elucidated.

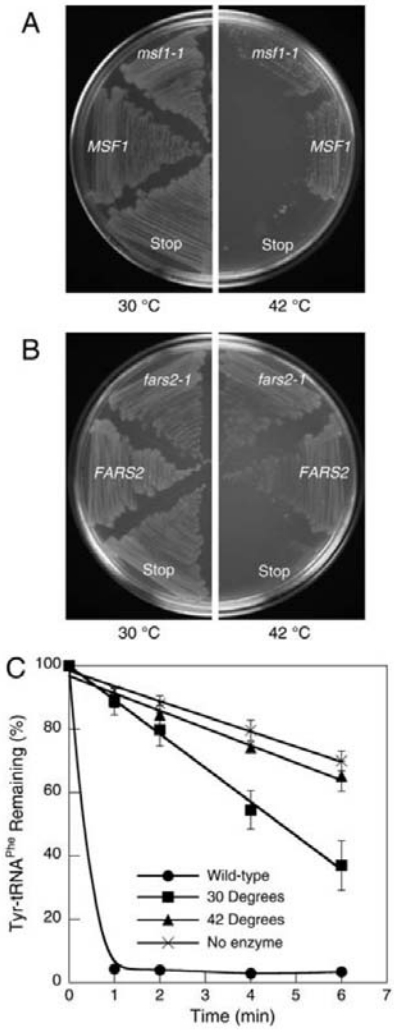

Fig. 3. Function of A333G mtPheRS in *E. coli*. Rescue of the growth phenotype of *E. coli* NP37 transformed with (*A*) yeast *MSF1*, *msf1-1*, or *msf1* with a stop codon at A333 and (*B*) human *FARS2*, *fars2-1*, or *fars2* with a stop codon at A308. (*C*) Transediting activity of *E. coli* wild-type and NP37 *FARS2* complemented cell extracts grown at 30 or 42 °C. Reactions were carried out at 42 °C. Data points are an average of three independent experiments with errors bars representing 1 SD.

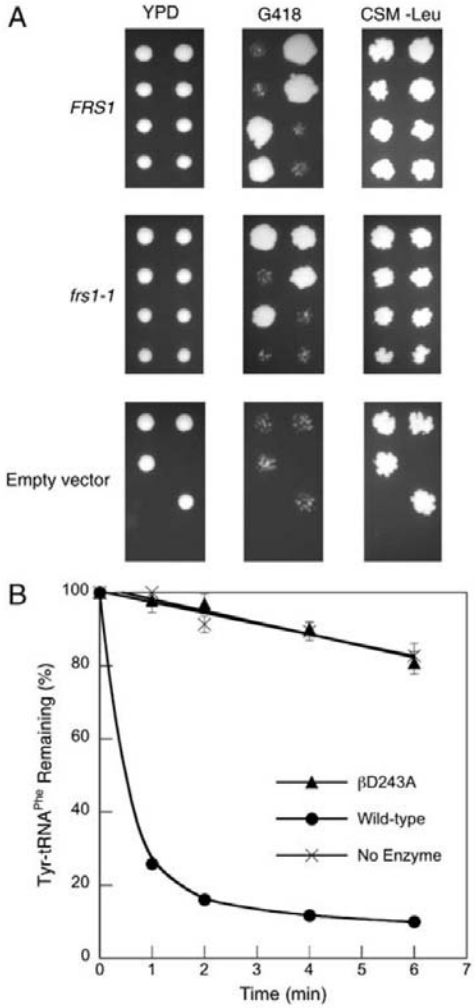

Fig. 4. Function of βD243A ctPheRS in *S. cerevisiae*. (*A*) Dissection of tetrads from *S. cerevisiae FRS1*/*frsΔ* complemented with pFL36-*FRS1*, pFL36-*frs1-1*, or pFL36 onto YPD and replica plated onto G418 and CSM-Leu. In liquid YPD doubling times for *frsΔ* pFL36-*FRS1* and pFL36-*frs1-1* were 1.37 ± 0.05 and 1.34 ± 0.09 h, respectively. (*B*) Posttransfer editing activity of *S. cerevisiae frs1Δ* pFL36-*FRS1* and *frs1Δ* pFL36-*frs1-1* extracts at 37 °C. Data points are an average of at least three independent experiments, with errors bars representing 1 SD.

Materials and Methods

Protein Preparation and Analysis. Proteins were prepared as described previously (12, 19). *E. coli* BL21-RIL/pET16b producing His6-tagged mtPheRS encoded by the MSF1 gene was a gift from R.A. Zimmermann (University of Massachusetts, Amherst, MA). The ctPheRS α and β subunits, encoded by the *FRS2* and *FRS1* genes, respectively, were expressed in tandem from pQE31- FRS-sc (producing His6-tagged WT ctPheRS) in *E. coli* BL21-RIL. *E. coli* pET- 21c(þ) encoding human mtPheRS (*FARS2*), producing mature His6-tagged wild-type PheRS, was a gift from L.L. Spremulli (University of North Carolina, Chapel Hill, NC). His6-tagged proteins were purified on nickel-nitrilotriacetic acid-agarose by standard procedures. Point mutations were introduced by site-directed mutagenesis using the Quikchange procedure (Stratagene). CD spectra were measured at 25 °C in an Aviv 62A DS spectropolarimeter (Aviv). The protein concentration was 5 μM in 50 mM Tris-HCl, pH 7.5, and 5% glycerol. CD spectra were measured from 200 to 250 nm (five scans per sample) with a step size of 1 nm in a 1 mm path length cuvette with 1 nm bandwidth and 5 sec averaging time. Protein-only spectra were obtained by subtracting the CD signal for buffer alone.

ATP-PPi exchange reactions were performed at 37 °C as described (19) with the exception that the amounts of amino acids used were 1.3 μM–1 mM Phe or 170 μM–6.8 mM Tyr, and the concentration of enzymes used was 5–150 nM ctPheRS, 150 nM mtPheRS, or 100 nM *E. coli* PheRS. To ensure the absence of Phe contamination, Tyr was subjected to several cycles of heating and cooling to remove any trace amounts of Phe (37). Posttransfer editing reactions contained 100 mM Na-Hepes, pH 7.2, 30 mM KCl, 10 mM MgCl2, 2 μM [^{14}C] Tyr-tRNA^{Phe}, and 74 μg (OD595) of *E. coli* crude extract or 0.006 U of *S. cerevisiae* extract, where one unit of PheRS corresponds to the amount of protein necessary to catalyze the formation of 1 nmol of Phe- tRNA^{Phe} min^{-1} at 37 °C. *E. coli* crude extracts were preincubated at 42 °C before addition to the reaction mixture. After the addition of crude extract, reaction mixtures were incubated at 37 or 42 °C, and the deacylation reaction followed bymeasuring the [^{14}C] Tyr-tRNA^{Phe} remaining in aliquots of 7 μL removed after 0–6 min of incubation.

Construction, Manipulation, and Growth of Yeast Strains. Strains derived from *S. cerevisiae* W303 (*MATa, ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1- 100*) were used to carry out all in vivo experiments with the exception of those used in test crosses. In vivo site-directed mutagenesis was performed by the *delitto perfetto* method (38). A counter selectable reporter (CORE) cassette was inserted into the wild- type *MSF1* gene of wild-type diploid *S. cerevisiae* near the A333 codon through homologous recombination resulting in the *msf1*::CORE strain. Upon introduction of the CORE cassette, cells were sporulated, resulting in 1:1 segregation of the cassette into the daughter spores. Haploid cells containing the *msf1*::CORE strain were *rho*^{0}. Recombinant *MSF1* with mutations created by quick-change site-directed mutagenesis was used to transform *msf1*::CORE *rho*^{0} haploid cells. Recombination of the mutated gene with the *msf1*::CORE led to excision of the CORE and insertion of the A333G mutation (msf1-1) into the chromosome. To recover mitochondria, the haploid W303 *msf1-1* strain was mated to a W303 wild-type haploid strain, resulting in a heterozygous *rho*$^+$ strain. The resulting diploid strain was sporulated to obtain haploid *msf1-1* cells. Insertion of the A333G mutation in W303 *msf1-1* was confirmed by sequencing. Haploid strains are referred to as W303 *MSF1* or W303 *msf1-1* depending on the genotype. W303 *msf1Δ* was created through the replacement of the *MSF1* open reading frame with a KanMX4 cassette by homologous recombination in a W303 *MSF1* homozygous diploid. W303 *msf1Δ* was then obtained by sporulation and dissection.

The presence of mitochondrial DNA was determined by crossing W303 haploid strains with KL14-4A/60 (*MATa, his1, trp2, rho*^{0}) or D27310B/50 (*MATα, ade5, rho*^{0}) (39) on minimal

glycerol medium followed by replica plating onto minimal ethanol/glycerol medium. To determine the initial state of the mitochondria W303 diploid cells were grown in either yeast extract/peptone/ dextrose/adenine (YPDA), sporulated on minimal sporulation medium (1% CH3COOK, 0.1% yeast extract, 0.05% glucose), and dissected directly onto ethanol plus glycerol medium and grown for 3–4 days at 30 °C.

S. cerevisiae strain YLR060W BY4743 (*MATa/MATα, his3Δ1/his3Δ1, leu2Δ0/ leu2Δ0, lys2Δ0/LYS2, MET15/met15Δ0, ura3Δ0/ura3Δ0, FRS1/frs1::kanMX4*) (ATCC) was used to carry out all in vivo experiments on yeast cytosolic pheRS (FRS1). A 3-kb genomic region including *FRS1* and its native regulators was cloned into the centromeric shuttle vector pFL36 and the D243A mutation (frs1-1) introduced through site-directed mutagenesis. YLR060W BY4743 was transformed with pFL36-*FRS1*, pFL36-*frs1-1*, or pFL36. Resulting strains were sporulated, dissected on YPD, and replica plated onto YPD with 200 μg⁄mL geneticin (G418) and complete supplement media minus leucine (CSM -Leu; Sunrise Science Products). Growth rates of haploid *frsΔ* strains complemented with pFL36-*FRS1* and pFL36-*frs1-1* were determined in duplicate in 250-mL flasks with 50 mL of YPD. Cultures were shaken at 225 rpm at 30 °C. Samples of 1 mL were taken every hour with growth monitored spectrophotometrically at an absorbance of 660 nm. Cell-free extracts were prepared the same as *E. coli* cell-free extracts with the exception the cells were grown in 100 mL YPD overnight, washed, and resuspended in 5 mL 100 mM Tris-HCl, pH 8.0, 5 mM 2-mercaptoethanol, 500 μM diisopropyl fluorophosphate, 500 μM phenylmethylsulfonyl fluoride. Cells were opened with 1 mL glass beads by vortexing for 2 min, 6 times.

E. coli pheS^{ts} *Complementation and Preparation of Cell-Free Extracts*. *E. coli* NP37 (26) was transformed with the mature human mtPheRS (*FARS2*) cloned in pET-21c(þ) and pRARE, which expresses six rare tRNAs (Novagene). Point mutations were introduced by quick-change site-directed mutagenesis with the Quikchange kit (Stratagene). Transformants were plated on LB supplemented with 100 μg⁄mL ampicillin, 30 μg⁄mL chloramphenicol, 0.4 mM IPTG at 30 or 42 °C for 48 h. Prior to preparation of cell-free extracts, revertants of NP37 were removed from plates grown at 42 °C for 48 h. The remaining cells were then removed from the plates and resuspended in 100 mM Tris-HCl, pH 8.0, 5 mM 2-mercaptoethanol, 500 μM diisopropyl fluorophosphate, 500 μM phenylmethylsulfonyl fluoride, washed once, and resuspended in the same buffer. Cells were sonicated at 70% output with a Sonifier 450 (Branson) equipped with a microprobe. The resulting extract was centrifuged at 100; 000 × g for 1 h. The soluble extracts were dialyzed overnight at 4 °C against 100 mM Tris-HCl, pH 8.0, 5 mM 2-mercaptoethanol, 1 mM ethylenediaminetetraacetic acid and concentrated in the same buffer plus 50% glycerol.

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